Immunological Applications of Microspheres

by
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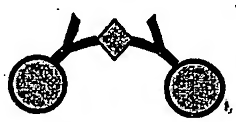
Introduction: Microsphere-based diagnostic tests (qualitative, yes/no results) and assays (quantitative results) are usually based upon the very specific interaction of antigen (Ag) with antibody (Ab). Sub-micron sized polystyrene microspheres, often called "uniform latex particles" are used for the solid support; Ab or Ag can be adsorbed onto them. These "sensitized" microspheres then act to magnify or amplify the Ag-Ab reaction which takes place when they are mixed with a sample containing the opposite reactant. In simple particle agglutination, a positive test results when uniformly dispersed milky-appearing Ab-coated particles in a drop of water on a glass slide react with Ag in a drop of sample (whole blood, serum, urine, etc.) to cause particle agglutination (clumping of the microspheres, to look like curdled milk) (Fig. 1). Similarly, an agglutination test for Ab can be made with Ag-coated particles.



Tests	Assays
Latex Agglutination Tests	Turbidimetric Immunoassays
(Slide, Tube or 96-well Plate)	Nephelometric Immunoassays

Where = MicroSphere, = Antigen (Ag), and = Antibody (Ab)

Figure 1 Agglutination Tests and Assays

Qualitative Agglutination (Tests): Microsphere or latex agglutination tests (LAT's) have been around since 1956, when Dr. Jacques Singer developed a rheumatoid factor test.[1] Since 1956, LAT's have been applied to >100 infectious diseases. Types and recent examples are as follows:

- Bacterial: leprosy, cholera, *Yersinia enterolytica*, Lyme Disease, TB
- Viral: HIV, *Herpes simplex*, cytomegalovirus
- Fungal: aspergillosis, candidiasis, cryptococcosis
- Mycoplasmal: mycoplasmal pneumonia (*Mycoplasma pneumoniae*)
- Protozoal: amoebiasis, toxoplasmosis
- Rickettsial: Rocky Mountain spotted fever

Commercial LAT's exist for >60 other chemical analytes, e.g., hCG, RF, CRP, ASO, FDP, and fecal occult blood. Many other applications in veterinary medicine (feline parvovirus and cryptococcosis), plant health (potato viruses), law enforcement (DAU, drugs of abuse in urine), food (antibiotics in milk) and the environment use LAT's for analyte detection.

LAT's are portable, rapid, efficient, and useful under even the most primitive conditions. Ideal for point-of-care use in the field, ambulance, or bedside, they can be run quickly and simply (2 minutes from sample preparation). Diagnosis and treatment can

Illumina Tech
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commence promptly, before the patient is transferred or discharged. Examples of such tests include those for FDP (fibrin degradation products), myoglobin (for heart attacks), rotavirus (to isolate contagious pediatric patients), and for sexually transmitted disease clinics (test patients and treat them before they leave).[2]

Tests for *new* analytes are continually being added, such as TechLab's Leuko-Test, an LAT for fecal leukocytes– actually a test for lactoferrin released from these leukocytes in diarrheal stool specimens. Ideal as a screening test for "traveller's diarrhea", it can diagnose inflammatory diarrhea caused by dangerous bacterial pathogens such as *Shigella*, *Salmonella*, *Campylobacter*, and *Clostridium difficile*, and it can reduce by >95% the number of samples which require culturing and follow-up.[3] By the way, this one was developed by an alumna of The Latex Course.

Another new development, from Eiken Chemical, is a *dual* test (two LAT's in one)– an occult blood test for hemoglobin and transferrin in feces. The microspheres have anti-hemoglobin *and* anti-transferrin bound to them, so that *either* hemoglobin or transferrin (or both) can cause agglutination. "Combining assays for both hemoglobin and transferrin gives ...a much higher detection rate compared to hemoglobin [alone]."[4]

Also new is an LAT for BFP in urine, a new tumor marker for bladder cancer.[5]

1996's newest LAT is for systemic lupus erythematosus (SLE), from Diatech Diagnostics.[6] It is a 3 minute test for antinuclear Ab's.

Murex's Staphaurex Plus™ latex, using beads coated with human fibrinogen and IgG, can agglutinate *three* different ways– by encountering either clumping factor, surface antigen, or protein A (found on most Staph A).

***Active* Agglutination Tests– Wet & white on black:** The earliest tests used *liquid* reagents made with plain, white microspheres and were run on washable, reusable glass slides, usually with a black background. Tests are now run on disposable plastic or coated paper cards. White slides are also available for colored microsphere tests. Most active LAT's require the clinician to rock the slide or card for 2-5 minutes to mix sample and reagent and to speed up agglutination.

Slide Test "Automation": Recent refinements of the "ordinary" slide test include novel devices designed to make the tests less technique-dependent. In Wampole's (Carter-Wallace) **Fast Trak™** (Fig. 2) and Roche's **OnTrak™** (Fig. 3) devices, the sample and reagent with coated microspheres are mixed and guided into a "track" or capillary. As the reactants move down the track by capillary action, they mix them-selves, and agglutination is read with transmitted light after they reach the end 2-3 minutes later. No hand rocking or rotation is necessary, and the test is quite operator-independent.

Note that the Roche DAU tests are run as inhibition tests, so a positive test yields *no* agglutination (*i.e.*, drug in urine inhibits the agglutination), while a negative test shows agglutinated microspheres.

Dried Microspheres: Several companies produce tests with reagent coated microspheres dried on a card. In use, the microspheres are rehydrated with sample, stirred, and observed for agglutination. Excellent shelf-life should be possible with dried reagents.

Figure 2 Wampole Fast Trak™ Slide with agglutinated particles in top track

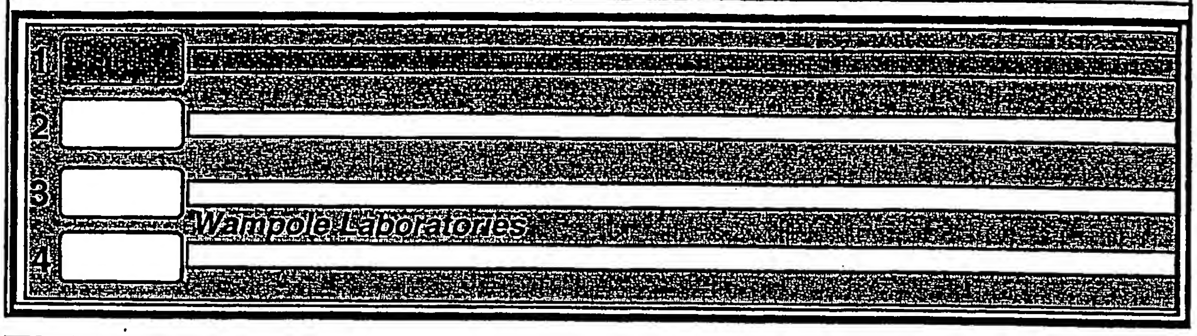
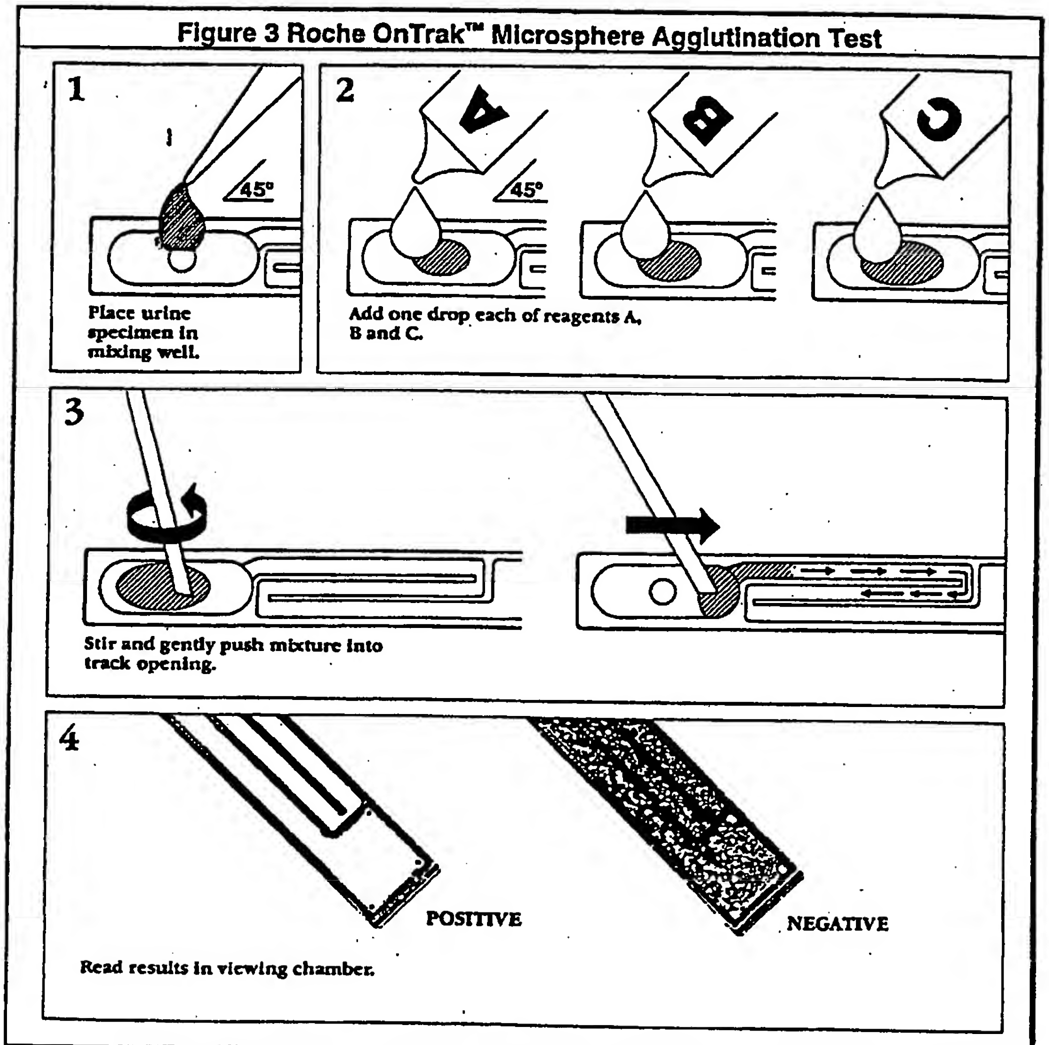


Figure 3 Roche OnTrak™ Microsphere Agglutination Test



Dyed microspheres provide different contrast (dyed microspheres observed against a white background). *Staphylococcus* tests by Carr-Scarborough (red microspheres) and REMEL (black) use dyed microspheres. REMEL also has a *black* LAT for *E. coli* 0157:H7 (the strain implicated in the 1993 Jack-in-the-Box restaurant food-poisoning outbreak). Two or three new mycobacterium LAT's (for *M. tuberculosis* and *M. avium* or *M. kansasii*) also use dyed microspheres. The *E. coli* and mycobacterium tests were developed by Latex Course alumni, at different companies.

Mixed Colors: MUREX has a *Salmonella* test which uses antibodies to *three* different antigen groups bound to three different colored microspheres (red, blue, and green). By comparing the color of the combined agglutinated microspheres to a background color, one can determine which salmonella groups are present in the sample. They also have a *Shigella* test kit employing two colors of microspheres and two reagents, to differentiate between four different strains.

See "Microspheres as Markers and Stains" for more uses of dyed microspheres.

Slide Test Sensitivity: Current LAT's generally use 0.2-1.0 µm diameter microspheres. My calculations show that larger microspheres should theoretically yield more sensitive tests; the assumptions are as follows:

- ~100 clumps must be seen to determine agglutination
- Each clump must be ~50 µm in size to be seen by eye
- ~10 bonds required per microsphere to agglutinate
- 10 µL sample size

If a single microsphere is 1 µm in diameter, then ~50^3 or ~10^5 microspheres will be required to make one visible clump. To figure out how much Ag or Ab is required to detect agglutination, multiply 10 bonds per microsphere x 10^5 microspheres per clump x 100 clumps, which equals ~10^8 molecules or 10^{-16} mole of agglutinator. If MW = 150,000 (≈ MW of IgG), then the necessary amount of Ag or Ab would be 15 pico-grams. If a 10µL sample is used, then sensitivity would be 10^{-16} mole/(10 x 10^{-6} L) = 10^{-11} mole/L = 10 picomoles/L. If microspheres are 0.1 µm, then sensitivity will only be 10 nM. If 10 µm microspheres are used, sensitivity improves by 1000X to 10 fM. The calculations are summarized below:

Microsphere	# of Microspheres in		Agglutinator Required...			*Calculated*
Diameter	1 clump	100 clumps	Molecules	Moles	wt., if IgG	Sensitivity
0.1µm	10^8	10^{10}	10^{11}	10^{-13}	15 ng	10 nM
1.0	10^5	10^7	10^8	10^{-16}	15 pg	10 pM
10.0	10^2	10^4	10^5	10^{-19}	15 fg	10 fM

If these calculations are close to being accurate, they lead to the prediction that you will get greater sensitivity by using larger particles. Please feel free to challenge this idea and report any results which would shed any light here.

Some limits to the theory are 1) clumps of larger microspheres may not be strong enough to withstand hydrodynamic forces trying to break them apart, and 2) it will take considerably longer for all the larger microspheres to form clumps. At an earlier course, Bob Veltri estimated that LAT's could have sensitivity of 25 ng with submicron particles, but what is possible with larger particles?

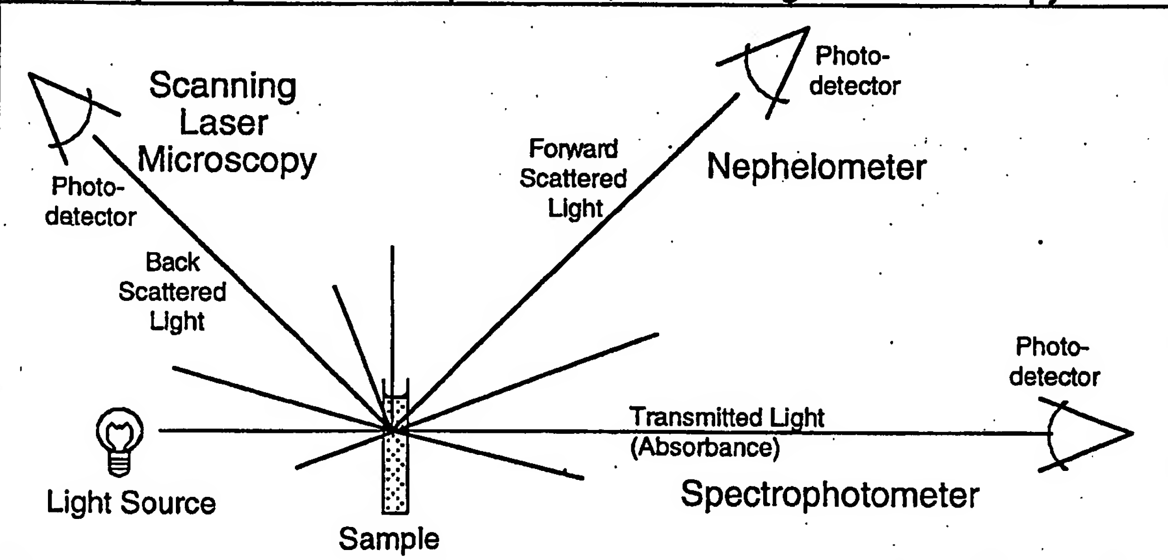
Passive agglutination tests (like hemagglutination tests): Classically, red blood cells (often "tanned" or specially processed to preserve them) have been used in agglutination tests. These *hemagglutination* tests are run by mixing samples with the coated RBC's in 96 well plates with "V"-shaped bottoms. They are called *passive* since there is no rocking involved. Agglutinated cells fall out of suspension and form a pink, lacy pattern covering the bottom of the wells; unagglutinated cells roll to the center of the "V" to form a dark red button.

RPLA (reverse passive latex agglutination) tests use microspheres to replace the RBCs. Dyed microspheres have been found to be more reproducible and shelf-stable than the RBCs. Tests using blue-dyed polystyrene microspheres have been made for 96-well plates,[7] and generally use microspheres which are larger (>1 μm) and heavier (>>1.05 g/mL) than those in regular LAT's.[8] Sawa International (Tokyo) has six RPLA tests for bacterial toxins which cause hemorrhagic colitis, hemolysis, toxic shock syndrome, food poisoning, and two other enterotoxins.[9]

Sonologics (Woburn, MA) has an instrument, developed at the University of Wales, which uses a *"focused ultrasound wave to accelerate agglutination."* Normal two hour hemagglutination tests take only five minutes (two minutes for ultrasonic treatment in their "black box" + three minutes to develop the pattern). "Eighty different agglutination tests have been identified for which the technology is applicable," including LAT's in capillary tubes.[10,11,12] Ultrasonics is claimed to increase sensitivity to 40 pg/mL of *Candida albicans* mannan and 70 pg/mL of *Aspergillus fumigatus* galactomannan for a 250X and 500X increase in sensitivity over conventional LAT's.[13]

Quantitative Agglutination (Assays): Spectrophotometers and nephelometers have been used for years to measure protein precipitation directly. When these instruments, which measure transmitted, absorbed, or scattered light, are used in place of the human eye, it is possible to quantitate agglutination and to develop sensitive microsphere agglutination immunoassays (Fig. 4).

Figure 4 Turbidity of a Microsphere Dispersion Measured with Spectrophotometer, Nephelometer, or Scanning Laser Microscopy



The intensity of light scattered by particles dispersed in water varies with the number of particles, the diameter of the particles, the wavelength of the incident light, the angle of the detector to the incident light, and a number of other variables. As agglutination starts, single particles first become doublets; thus the number of light scatterers drops dramatically (decreases by half), and the apparent diameters increase rapidly up to 2X. After this point, the changes in numbers and diameters are less rapid. Microsphere immunoassays can be very sensitive, since the change of scattered light intensity is highest at the very beginning of agglutination or at the lowest concentrations of analyte.

Microspheres which scatter light best have diameters approximately equal to the wavelength of light being scattered. Therefore, for visible light (λ= 390-760nm) the best scattering microspheres have diameters of 390-760nm (0.39-0.76µm). Microspheres outside this range will not scatter as well. In practice then, one can start with microspheres <0.1 µm which are poor scatterers. As they agglutinate, the clumps quickly grow to a size where they scatter light much better. Thus, change of scattered light *vs.* analyte concentration can be the basis for very sensitive end-point or rate method immunoassays. UV light requires smaller microspheres (<<100nm) and infrared light can use ~0.5 µm microspheres. Conversely, one can also start with microspheres which scatter well (perhaps 0.5 µm microspheres) and observe them clumping to sizes where they do not scatter as well, but most assay systems seem to use the principle of small, poorly-scattering microspheres clumping to form big, highly-scattering clumps.

One can use a nephelometer to follow scattered light directly[14,15] or a spectrophotometer to measure change of "absorbance" of light (measure scattered light indirectly).[16,17] DuPont calls these techniques PETIA and PETINIA for particle enhanced turbidimetric immunoassay and particle enhanced turbidimetric inhibition immunoassay.

Behring's "latex-enhanced" nephelometric method measures forward scattered light of 840nm at ~20° angle from the light beam. Their assay for C-reactive protein (CRP) was judged good enough to have been proposed as *the reference method* for this assay.[18]

Modern 96-well plate readers can read a complete plate in about two seconds and can be used for end point or kinetic assays. One proposed lactogen assay measures microsphere agglutination turbidimetrically in such a plate reader.[19]

These instrumental methods have now been applied to a wide variety of commercial assays,[20] and new assays and even new analytes are being added continually. This is truly one of the steady growth areas for the microsphere business. Orion Diagnostica (Finland) has a compact but sophisticated "doctor's office" size turbidimeter for microsphere assays. They are steadily adding new assays.

The Binding Site has a new nephelometric immunoassay for β_2-microglobulin using microspheres, and Instrumentation Laboratories has a new turbidimetric (spectrophotometric) assay for the same analyte for its Monarch centrifugal analyzer. (This analyte is an important prognostic indicator for AIDS.) Dako AS (Denmark) recently introduced a new "particle enhanced turbidimetric" (PET) assay for cystatin C– a new analyte described as a "better marker than serum creatinine for glomerular filtration rate."[21]

Spectrophotometric/nephelometric sensitivity should be better than LAT sensitivity and may be as good as 100 pg/mL (1 pg/10 µL) for proteins.[22] Zolg estimated turbidimetric

sensitivity at 5×10^7 molecules (~10^{-16} moles =100 attomoles),[23] which would be ~1.5 pg/10 µL [if MW =150,000 (as for IgG) and 10 µl sample volume were used]. He also estimated "quantitative agglutination" (like ACADE did, see next section) at 5×10^6 molecules, or one order of magnitude better than turbidimetry. **Thus, the turbidimetric *assay* is estimated to be at least 10X more sensitive than a typical 1 µm microsphere LAT (see above), and perhaps 10,000X more sensitive than a LAT test using 0.1 µm microspheres.** [Dako[21] (previous paragraph) claims turbidimetric detection limit of 0.15 mg/L (=1.5 ng/10 µL).]

The work by Price, Newman, *et al.*,[16,17] has prompted interest in very small microspheres with higher refractive indices. For polyvinylnaphthalene, n_D =1.68 *vs.* 1.59 for polystyrene (n_D = refractive index using light at sodium D line or 589.26nm). These "brighter" microspheres scatter light better, especially when they agglutinate to the optimum scattering size. One can also get a higher refractive index for polystyrene by using shorter wavelength light: n_{400nm} = 1.63.[24]

In another case, researchers chose "duller" microspheres of polybutylmethacrylate (n_D = 1.43), apparently because they do *not* scatter as well.[25] This property may be important in some instruments, for lower background scattering (a lower blank value for the calibration curve).

Other Instrumental Agglutination Methods:
Scanning laser microscopy instruments have been proposed to quantify agglutination.[26] This new method might offer advantages since, by using larger microspheres (>1 µm) and operating with concentrated microsphere suspensions, agglutination may occur more quickly.

Angular anisotropy (or "two-angle light scattering")[27,28] and **quasi-elastic light scattering** (also called "dynamic light scattering" or "photon correlation spectroscopy")[29] are more powerful techniques which have been investigated and patented for assay systems. These more sophisticated methods *might* deliver better sensitivity and should be considered after simpler methods have been exhaustively explored.

Sensitivity 10-15 times better than turbidimetry was claimed for work by Technicon and later by ACADE. They used **particle counters** to measure changes in numbers of single particles or clumps of particles during agglutination.[30]

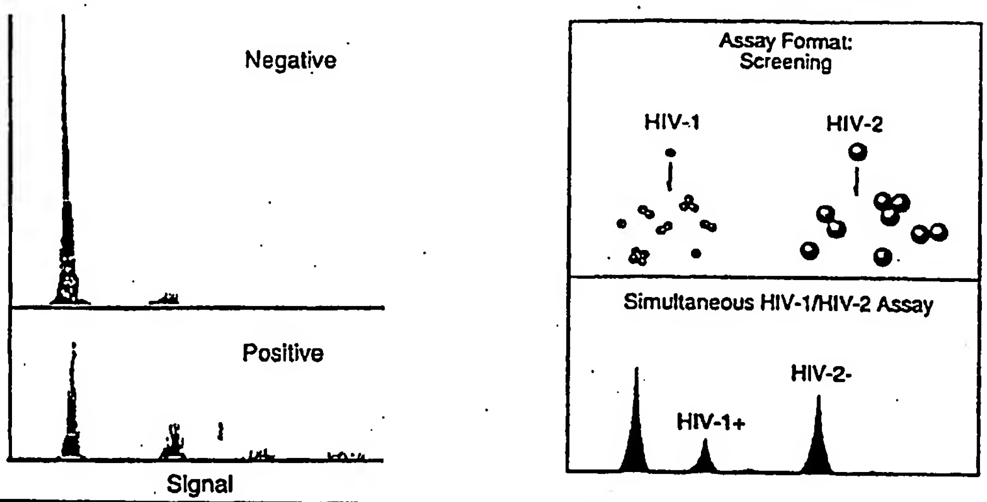
Sienna Biotech's Copalis™ technology uses "optical sizing flow particle analysis", a sensitive laser-based particle sizer/counter. They count single, Ab-coated microspheres (~1 µm in diameter) before and after agglutination. As single particles become agglutinated by antigen in a sample, the signal in the single particles channel decreases, while the counts increase in the doublet, triplet, etc. channels. Thus, a *decrease* in single particles is proportional to analyte concentration. They can do several tests simultaneously using different sized microspheres coated with different Ab's, and can use whole blood with no interference from cells. They claim a sensitivity for TSH = ~1 pM. (Fig. 5)

Fujirebio Inc. introduced special microsphere-based reagents and an instrument to read agglutination by **pattern recognition.** It scans the bottoms of 96-well plates and detects the difference between a central button of non-agglutinated microspheres and the typical diffuse, lacy pattern of agglutination. Researchers at the University of Wales are developing a similar instrument.[10,11,12,13]

Figure 5 Sienna Biotech's Copalis™ Technology

a) Single particles peak decreases as agglutination occurs

b) Simultaneous assays done on different sized particles



In **fluorescence quenching analysis,** fluorescent microspheres yield a lower fluorescent output on agglutination. When such microspheres clump together they interfere with and absorb light from each other so that less light gets to the detector.

Magnets have been used to accelerate agglutination (pulling the particles together) in fluorescence quenching caused by agglutination of fluorescent magnetic particles.[31]

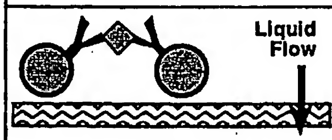
Magnetic microsphere agglutination can be measured by **magnetic moment analysis.** As the microspheres pass through a magnetometer, agglutinated microspheres give a larger signal than single microspheres, so agglutination can be quantified (Hitachi patent[32]).

Magnetic particles can be coated with one Ab and mixed with plain particles coated with another Ab to the same Ag, then combined with a sample. If Ag is present in the sample, a magnet can be used to remove coagglutinated particles. The absorbance of the suspension should be very different before and after sample addition.[33]

Agglutination Made Colorimetric? Indicia (France) offers "Spherotest,...a diagnostic system [for β_2-microglobulin] based on quantitative microagglutination of calibrated and sterically stabilized synthetic microspheres." "U.V.-visible absorption" results are read in a microplate reader.[34] Microspheres of diameter, d, dispersed in a liquid with closely matched refractive index will not scatter light, but will instead absorb a maximum amount of light at wavelength $\lambda_{max} \approx d/2$. [*e.g.*, 740 nm microspheres absorb at $\lambda_{max} = 340$ nm (blue) and appear yellow.] Upon agglutination, clumped particles appear as a different color and the loss of single microspheres results in a drop in $A(\lambda_{max})$ or absorbance at λ_{max}. Thus, a plot of $A(\lambda_{max})$ vs. analyte concentration yields a linear curve of >5 orders of magnitude![35]

Beyond Simple Latex Agglutination...

Filter Separation Agglutination Tests (& Assays): Kodak's earliest Surecell test kits used dyed agglutinated microspheres caught on a filter. Red microspheres coated with Ab were incubated with a sample and poured onto a filter. Single microspheres passed through the filter and no color appeared on the surface. If the sample contain-ed the appropriate Ag, the microspheres agglutinated, and the agglutinated clumps were caught on the filter, resulting in a red (or pink) positive color test for the Ag (Fig. 6).



	Tests	Assays
	Agglutination (Coagglutination) and Capture by Filter	(Quantifiable with reflectometer or strip reader.)

Figure 6 Filter Separation Agglutination Tests (& Assays)

Carter-Wallace's home pregnancy test, First Response®, uses plain microspheres (~1 µm) and very small (<50 nm) red gold sol. *To prepare the test:* the gold particles are coated with one antibody (**Ab_1**) to hCG (human chorionic gonadotropin); the plain microspheres are coated with an antibody to another hCG epitope (**Ab_2**); then the particles are mixed and lyophilized. *To use the test:* the particles are redispersed with a sample of urine. If the sample contains hCG, the particles are coagglutinated, yielding red clumps. The mixture is poured through a filter which catches the red clumps to yield a pink-colored filter. With negative urine, unagglutinated red particles pass through the filter and no color develops.

The principles employed in these two *tests* could easily be applied to *assays* where the reflected color intensity (as measured, perhaps, in a dry strip reader?) would correlate with the sample's Ag content. Such assays would be comparable to the ELISA's below, but simpler to operate, and probably more stable (no enzymes).

Akers Research has a new series of tests where black dyed antibody-coated microspheres are mixed with sample and poured onto a strip. If no antigen is present, the microspheres migrate up the strip where a black (grey) color is observed. Thus, if color is observed, it means a "negative" test result. But, if the microspheres are agglutinated by the sample, then the clumps are too large to travel up the strip, and no color develops at the observation point (no color = "positive").

Particle Capture Enzyme Linked ImmunoSorbent Tests and Assays: *To prepare these tests:* Ab_1 is bound to microspheres, and the microspheres are caught on a filter and dried (Fig. 7). *To use:* 1) a sample is first passed through the filter, and any Ag is caught by Ab_1 on the microspheres. 2) Ab_2-enzyme reagent is put through the filter; Ab_2 is caught by the Ag-Ab on the microspheres to complete the sandwich. 3) Enzyme substrate is passed through the filter and the reacts with enzyme to create an *insoluble* colored product (on the filter) which is proportional to the amount of Ag caught.

Various *tests* (like hCG, Strep A, and others) using this principle have been made by Hybritech (ICON), Abbott (Test Pack), Novo Nordisk,[36] IDEXX, and others.

	Tests	Assays
	Particle Capture ELIST	**Particle Capture ELISA**

Figure 7 Particle Capture Enzyme Linked ImmunoSorbent Tests & Assays

Murex's SUDS clinical tests use *wet* reagents (microspheres in suspension). Microspheres are captured on a filter *after* the sandwich reaction. Ab-coated microspheres + Ag (from sample) + second Ab-enzyme conjugate are mixed, then poured through the filter device to capture the microspheres, which are then reacted with an enzyme substrate to form color.

Assays have been made by Hybritech (ICON QSR), Abbott (IM_X and A_XSym), Neogen (Reveal), and others. Using a reflectance meter, the colored spots caused by analyte are compared to a blank and a standard spot to yield a true quantitative assay. Reveal is literally a "field" instrument (pocket-sized) for measuring plant pests, like fungal infestations of soybeans, and turf diseases on golf courses. A similar hand-held instrument would permit point-of-care quantitative assays in human diagnostics.

Strategic Diagnostics has a "Competitive Latex Enzyme Immunoassay" for industrial chemicals. Petroleum products (benzene derivatives) and explosives in soil and ground water are detected at parts per billion concentrations. Ab-coated 3 µm microspheres are first caught on a 1 µm filter. (Fig. 8) Then a sample is passed through the filter, and Ag, if present, binds to the Ab. Next, an enzyme/Ag conjugate is passed through the filter. If there was no Ag in the sample, the Ag/enzyme will bind to the filter, and added enzyme substrate will result in color on the filter. If there was Ag in the sample, *no* enzyme will bind to the filter; and there will be *no* color on the filter from the added enzyme substrate.[37]

Costar Corp. has proposed a microsphere agglutination capture ELISA scheme.[38] After reaction with a chromogenic substrate, soluble color product is measured in a microplate reader.

Dyed Microsphere Sandwich Test (also called "One Step", Chromatographic, or Strip Tests): In 1988, a new over-the-counter pregnancy test (Clearblue Easy®, developed and patented by Unipath) revolutionized diagnostic immunological tests. The test uses dyed microspheres in a sandwich format to give a one step test eliminating the need for unstable, color-generating enzymes[39] (Fig. 9).

To prepare the test: small (dark-blue) dyed microspheres (O) are first coated with primary antibody (**Ab_1**) to hCG; the microspheres (O-**Ab_1**) are dried on one part of a nitrocellulose strip; a secondary antibody (**Ab_2**) to hCG is immobilized on another section of the strip (**Ab_2–I**). *To use:* the strip is wetted at one end with urine. (Fig. 10) As the urine moves by capillary action, it picks up the blue microspheres (O-**Ab_1**), and carries them downstream; any hCG in the urine reacts with **Ab_1** on the microspheres (O-**Ab_1**-hCG). When the flow reaches the immobilized **Ab_2–I**, the dyed microspheres with hCG (O-**Ab_1**-hCG) are captured by **Ab_2–I** to form a blue line caused by the hCG sandwich (O-**Ab_1**-hCG-**Ab_2**–I). The blue line signals a positive pregnancy test.

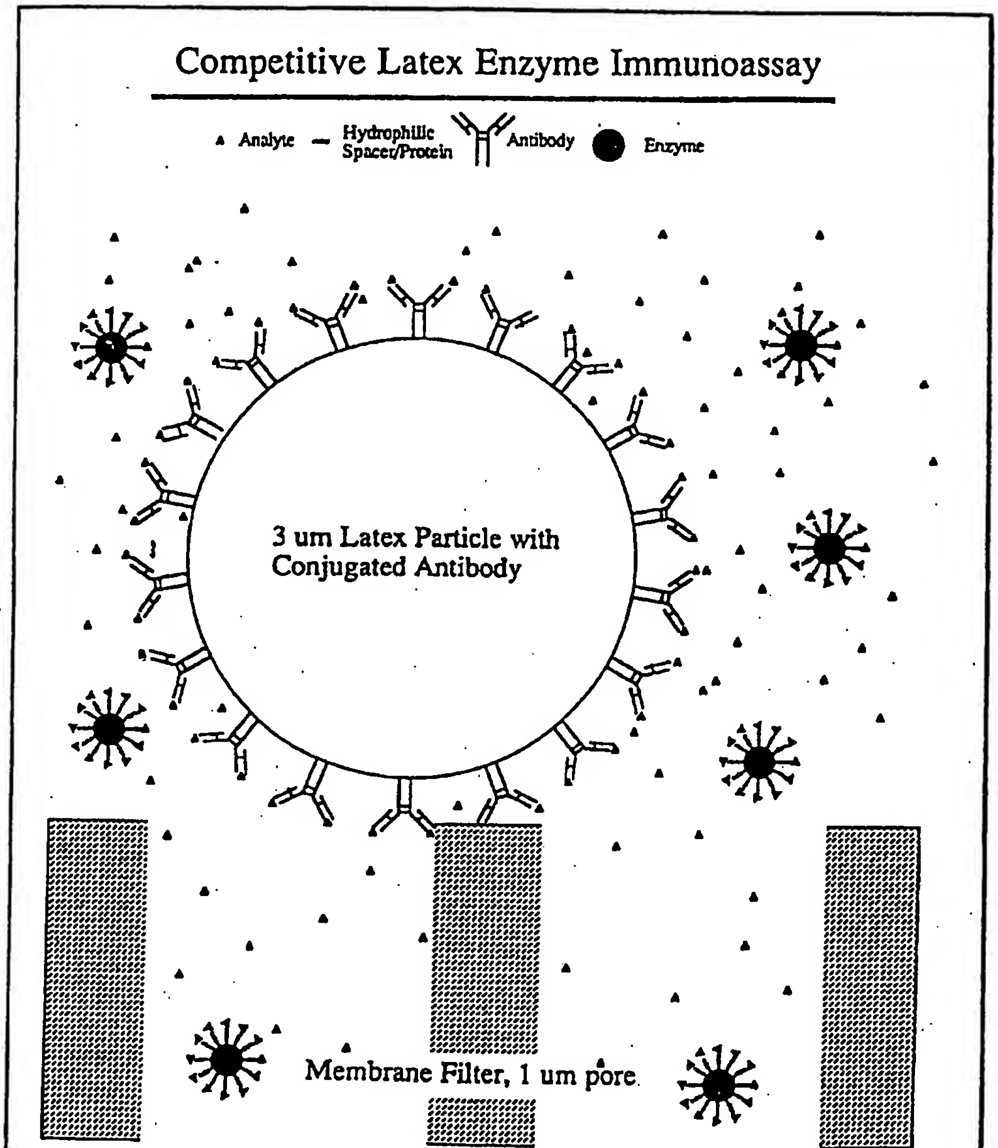


Figure 8 Strategic Diagnostics Assay for Water & Soil Pollutants

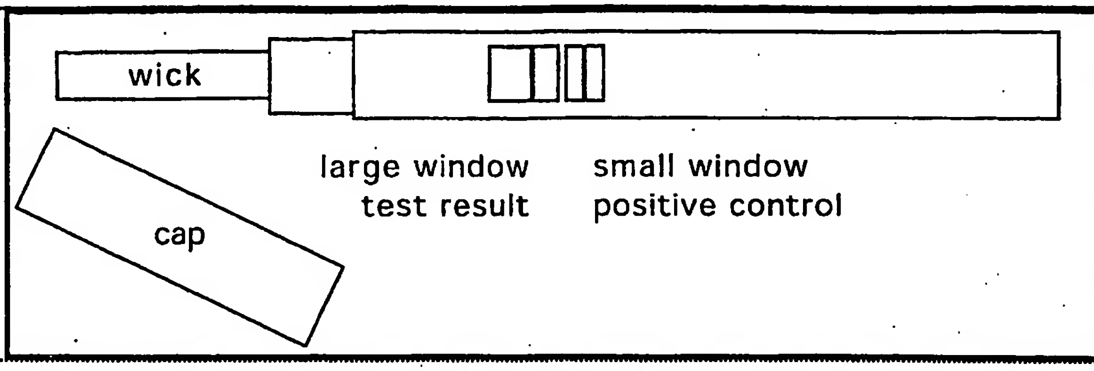


Figure 9 UNIPATH HOME PREGNANCY TEST

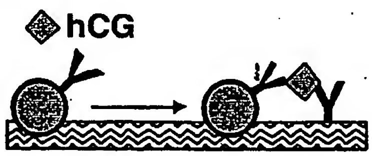
	Tests	Assays
	**Chromatographic Strip Tests**	**No known assay**

Figure 10 "One Step" Strip or Chromatographic Tests and Assays

Further downstream, there is another line of immobilized protein (**Ab_3**–I) which catches unconjugated O-**Ab_1** (as O-**Ab_1**-**Ab_3**–I) (independent of hCG) to form another blue line, which acts as a positive procedural control. If the second line does not form, the test results are invalid.

Other home tests– for LH (luteinizing hormone)– and *clinical* tests for Strep A, and Chlamydia are also available. Since the Unipath test, many other companies now offer laboratory single-analyte tests using this chromatographic principle. Examples are hCG, "popular" infectious diseases, and DAU[40] (Table 1). Eiken's new hemoglobin test claims 50 ng/mL sensitivity.[41,42] In March, 1996, Quidel began shipment of its QuickVue *H. pylori* strip test. It is the first one-step, quick diagnostic for *H. pylori*-positive ulcer patients, and the first strip test, that we know of, which uses *whole blood!*

In 1992, Biosite Diagnostics introduced Triage™, an eight analyte DAU test panel.[43,44] It is an *inhibition* test panel. In the *first* step, urine is used to reconstitute and react with a mixture of eight pairs of reagents. Each pair consists of very small colored (gold sol) particles conjugated to a drug (O-drug) + excess antibody (**Ab_1**) to that drug. In the *second* step, the urine and reagents mixture is allowed to migrate on a strip, whereon eight other antibodies to the eight drugs are bound in different locations, **Ab_2–I**. If the urine is drug-free, then **Ab_1** will bind all O-drug and none will be available for the second (solid-phase) reaction:

O-drug + xs **Ab_1** + **Ab_2–I** → O-drug-**Ab_1** + **Ab_2–I** (*no* color)

If a drug is present in the urine above a certain cut-off level (controlled by the amount of excess **Ab_1** present), then O-drug will be free to migrate along the strip to become

Table 1 Examples of Dyed Particle Chromatographic Strip or Sandwich Tests

Analyte	Type	Company (Test Name)
hCG	OTC	Unipath (Clear Blue Easy™ or Clear Blue One Step™), Carter Wallace (One Step™ paddle, First Response 1-Step), Johnson & Johnson (Fact Plus™, OEM by Abbott)
hCG	Lab	Unipath (ClearView™), Abbott (Test Pack Plus™) Pacific Biotech/Hybritech/Quidel (CARDS$^+$- OS™ & Concise™) Sinovus
hLH	OTC	Unipath (Clear Blue), Quidel (Conceive™)
Strep A	Lab	Unipath (ClearView), Abbott (Test Pack Plus) Pacific Biotech/Hybritech/Quidel (CARDS$^+$- OS & Concise)
Strep B	Lab	Pacific Biotech/Hybritech/Quidel (CARDS$^+$- OS)
Chlamydia	Lab	Unipath (ClearView)
Mononucleosis	Lab	Pacific Biotech/Hybritech/Quidel (CARDS$^+$- OS)
Rotavirus	Lab	Sinovus
H. pylori	Lab	Quidel (QuickVue™, whole blood ulcer diagnostic)
DAU tests	Lab	Drug Screening Systems (several analytes) Biosite (Triage™, panel of 8 drugs)
Hemoglobin (Occult Blood)	Lab	Eiken (OC- Hemocatch Eiken)
Brown Rot (wood decay)	Field	U.S.Dept. of Agriculture
Murine Ab type	Lab	Boehringer-Mannheim (ISO Strip™ mAb isotyping kit)
CPV (Canine parvovirus)	Vet Lab	Sinovus
FeLV (feline leukemia virus)	Vet Lab	Sinovus

bound by Ab_2–I. If a colored line is found, then that drug was present in the urine at a level above the threshold level:

$$\bigcirc\text{-drug} + \textbf{free drug} + \text{xs } Ab_1 + Ab_2\text{–I} \rightarrow \textbf{drug}\text{–}Ab_1 + \bigcirc\text{-drug-}Ab_2\text{–I} \text{ (color spot)}$$

An innovation in the Carter-Wallace First Response® 1-Step over-the-counter pregnancy test is the use of some Ab-coated larger microspheres on the membrane in the second and third Ab positions. (Fig. 11, shows only second Ab line). These large microspheres are too large to move on the strip and therefore act as anchors to hold the second (and third) Ab stripe from moving with the liquid flow.

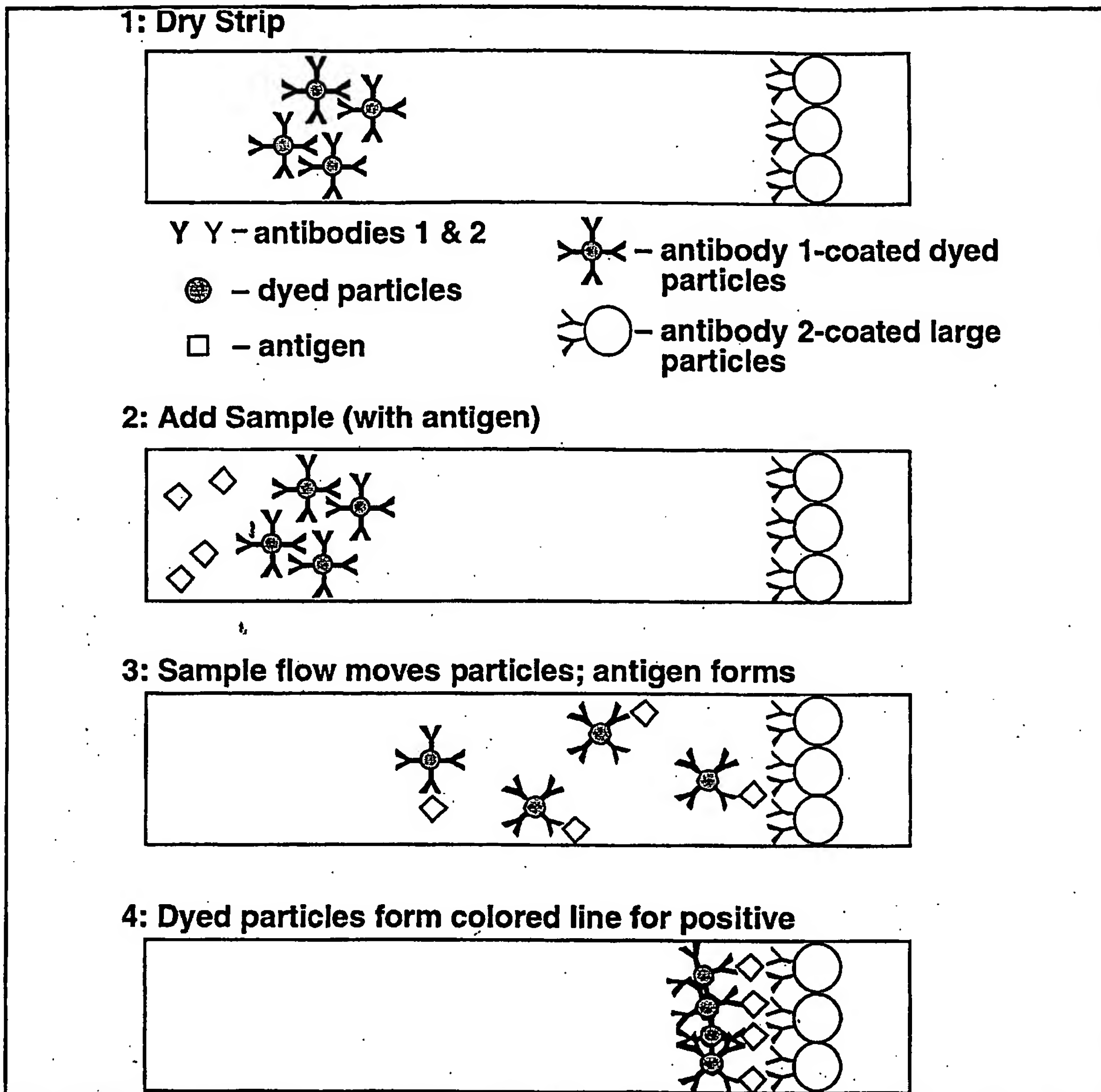


Figure 11 Strip Test Using both Dyed and Large Immobile Microspheres

The strip format is also being applied to non-human diseases, such as the USDA's recent test for brown-rot decay in wood, which detects six different fungi which attack wood. Not fancy, this test was home-made by another Latex Course alumna using polyester cloth as the strip and Ab's specific to brown rot. It is the first immunological *field* test for detection of brown rot.[45]

Boehringer-Mannheim has a test for murine antibody typing. This simple-looking, but sophisticated strip is useful for isotyping mouse mAb's and their light chains. On two sides of the strip, two bands will appear (out of eight possible bands plus two positive control bands) for Ab class and sub-class (IgA, IgG1, IgG2a, IgG2a, IgG3, IgM) and light chain (κ or λ).

Another Latex Course alumnus at Sinovus (Sweden) has commercialized two *veterinary* strip tests: for CPV (canine parvovirus) and FeLV(feline leukemia virus), as well as tests for hCG and rotavirus.

Sensitivity of Strip Tests: We calculated this, based upon these assumptions:

1) The minimum line dimensions for visibility of the blue line are perhaps 0.5 mm (500 µm) wide, 5 mm (5000 µm) long and 10 microspheres deep. With 0.25 µm microspheres, the line would be 500 µm/ 0.25 µm = 2000 microspheres wide; 20,000 microspheres long and 10 microspheres thick. Then, 2000 x 20,000 x 10 = 4 x 10^8 microspheres (~7 µg) per test.

2) It might take ~10 molecules of sandwich analyte (like hCG) reacting with each antibody-coated *dyed* microsphere and the second antibody immobilized on the strip in order to bind the dyed microspheres to the strip, or

$$\bigcirc\text{-Ab}_1 \quad + \quad 10\text{ hCG} \quad + \quad \underset{\text{(Ab bound to strip)}}{\text{Ab}_2\text{–}|} \quad \rightarrow \quad \underset{\text{(sandwich)}}{\bigcirc\text{-Ab}_1\text{–(hCG)}_{10}\text{–Ab}_2\text{–}|}$$

Some hCG will be wasted by binding to the wrong side of the dyed microsphere (the side away from the Ab_2 strip). In addition, it might require a ten-fold excess to bring about the reaction (90% of the hCG will be wasted and will not get to the microspheres or the strip-bound antibody).

3) It would probably take ~1mL to thoroughly wet one of these strip tests and to move the particles to and past the immobilized Ab stripe.

Therefore, sensitivity = 4 x 10^8 x 10 x 10 = 4 x 10^{10} molecules (~6.7 x 10^{-14} moles) to cause a positive reaction. This is equivalent to (or, sensitivity *could be)* ~67 pg/mL for a positive test, if MW = 1000 or 0.67 ng/mL, if MW = 10,000.

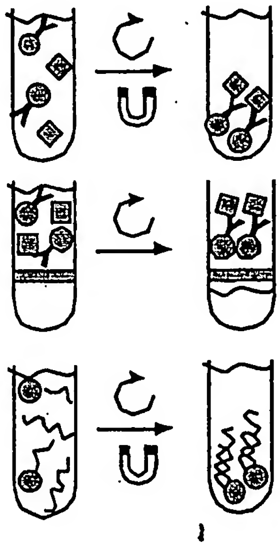
Please feel free to challenge these assumptions and recalculate the possible sensitivity.

Independently, strip test sensitivity has been estimated at 0.1-0.2 fmol/mL for direct test and 1-2 fmol/mL for competitve tests.

Stability and ease of use are important features of these tests. Since no enzymes are used, the dried products should be stable for years– as long as freeze-dried IgG is stable. The tests are so easily run that one can conceive of many "dip and read" field tests. How about farmers diagnosing a wide variety of plant diseases, literally, "in the field"[46]? Water tests for pollutants? Perhaps tests for *E. coli* in or on beef for FDA inspectors?

All of these tests also have the promise of becoming true *assays*. If the intensity of color formed could be read by a dry strip reader, for example, then a quantitative result can be obtained. Different colors of dyed microspheres could be used and different analytes color coded.

Solid Phase Assays (requiring solid/liquid separation):
Microspheres have unique properties– small enough to remain suspended for hours or longer at normal gravity, yet easily separated from suspension with a centrifuge, magnet, or filter. They have been used for years as solid supports for radioimmunoassays and other newer assays where solid/liquid (bound/unbound) separation is needed (Fig. 12, Table 2).



Assays

Solid Phase Immunoassays with Centrifugal or Magnetic Separation (includes cell separation or concentration)

Solid Phase Immunoassays with Filtration Separation

DNA/RNA Adsorption onto Silica MicroSpheres or Oligo DT Covalently Bound to Magnetic or Silica Particles which Captures Poly A Tail of DNA Probe or mRNA

Figure 12 Solid Phase Assays (requiring solid/liquid separation)

In a typical solid phase separation assay– for cardiac specific isoenzyme, lactate dehydrogenase, LD-1: antibody (D.8.1) is adsorbed on 0.8 µm microspheres; the microspheres are mixed with serum; D.8.1 binds interfering isoenzymes LD-2, 3, 4, & 5; microspheres are centrifuged to remove the competing isoenzymes; and free LD-1 is left in solution to be reacted with substrate and measured in a spectrophotometer *without* interference.[47]

Table 2 Microsphere Use in Assays with Solid/Liquid Separation

Microsphere Type	Separation Method	Assay Examples
Large PS(>0.8 µm)	Centrifugation	LDH-1 (Washington Univ.)
Smaller PS(<0.5 µm)	Centrifugal Filtration	LDL Separation (Genzyme)
Silica	Centrifugation	DNA/RNA Separations
<1µm PS/ fluor. tag	Filtration	PCFIA (IDEXX Screen Machine)
Superparamagnetic	Magnet	chemiluminescent immunoassays (ACCESS / SANOFI) mRNA Purification (Novagen) PCR/QBC/LCR DNA assays (IGEN, Gene-Trak, & Abbott) Template Prep/DNA Sequencing (MIT)

Genzyme's "Direct LDL [Low Density Lipoprotein] Cholesterol Immunoseparation Reagent Kit" uses "...latex beads coated with affinity purified goat polyclonal antisera to specific human apolipoproteins, which facilitate the removal of high density lipoprotein (HDL) and very low density lipoprotein (VLDL) in the specimen."[48] To use the kit, one mixes serum or plasma plus reagent (containing Ab-coated microspheres) and incubates in a separation device. After centrifuging the device (12,000 G's, 5 min.) to

filter the microspheres from the liquid, LDL cholesterol in the filtrate is measured using a conventional enzymatic cholesterol reagent.

Uniform *silica* microspheres will adsorb DNA or RNA to purify samples for PCR or assays. By adding chaotropic agents to nucleic acid solutions, the DNA/RNA can be made to adsorb onto silica. The density of the silica microspheres, 2.1g/mL, makes them easy to centrifuge. One can also covalently bind to surface modified silica[49].

PCFIA: In particle concentration fluorescence immunoassay, particles coated with one antibody trap a second antibody which traps an antigen or fluorescent-labeled antigen in a competitive binding assay. The particles are caught on a filter in the IDEXX "Screen Machine" and their fluorescence is measured. An internal assay from Eli Lilly & Co. for tylosin (veterinary antibiotic) in animal feeds is an example (Fig. 13).[50]

Kodak researchers have covalently bound oligonucleotide probes onto 1 µm microspheres and immobilized the microspheres in discrete locations on a membrane surface to capture biotinylated, PCR-amplified sample DNA. Each spot captures a different PCR sequence. These steps are followed by treatment with avidin-horseradish polymerase, a wash step, and dye-precursor. The result is colored spots which are diagnostic for specific DNA markers for various infectious diseases.[51]

The range of bead sizes for solid phase assays extends from <1 µm to >100 µm– the latter quite large by most standards. Sapidyne offers KinExA™, a new immunoassay instrument based on the kinetic exclusion assay method. Typically, 100 µm PMMA beads are coated with Ab or Ag. They are pumped into a flow cell built into the lens of a fluorescence analyzer and held in place by a screen for the duration of the reaction, then back-flushed out of the cell to complete the cycle.[52]

Large, Ab *fragment*-coated polystyrene beads have been used to collect bacteria from milk, water, and food. When using single chain Ab adsorbed onto polystyrene beads packed into a column, >90% of Pseudomonas was removed from a sample.[53]

Superparamagnetic Microsphere Based Assays: "Magnetic" particles permit fast and easy separation of solid and liquid phases. Actually *superparamagnetic*, the particles *respond* to a magnet but are not magnets themselves and retain no residual magnetism after removal of the magnet.

Magnetic particles are most commonly used in commercial solid phase RIA's, ELISA's, and newer chemiluminescent assays by Amersham, Ciba-Corning, and Biotrol.[54] The newest of these is Sanofi's ACCESS instrument using magnetic microspheres with chemiluminescent assays (first instrument sold in Spring of 1993).[55] Over a dozen Sanofi papers have now appeared; one example is their method for ferritin[56] (Fig. 14). Immunoradiometric assays (IRMA's) can also be done with magnetic particles.[57]

Reference Diagnostics (Bedford, MA) adds magnetic particles to the conventional dextran sulfate-$MgCl_2$ reagent for HDL cholesterol separation from samples permitting more rapid HDL cholesterol assays.

Both animal and plant cells, as well as cellular components, are separated using magnetic microspheres. DYNAL sponsors much of the animal work, and there are many papers, for example.[58],[59] More recently, magnetics were used to sort and collect

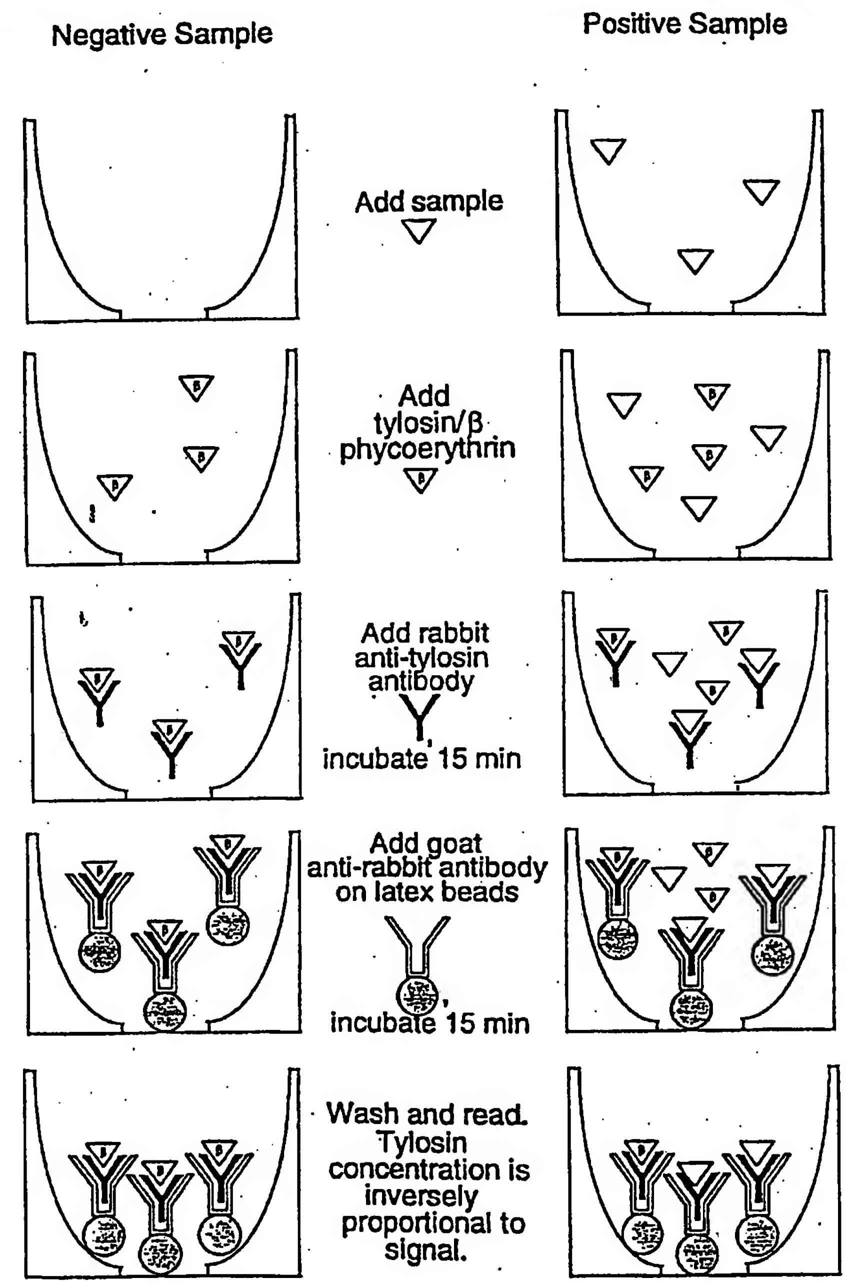


Figure 13 Screen Machine™ Fluorometric ImmunoAssay for Tylosin (Lilly)

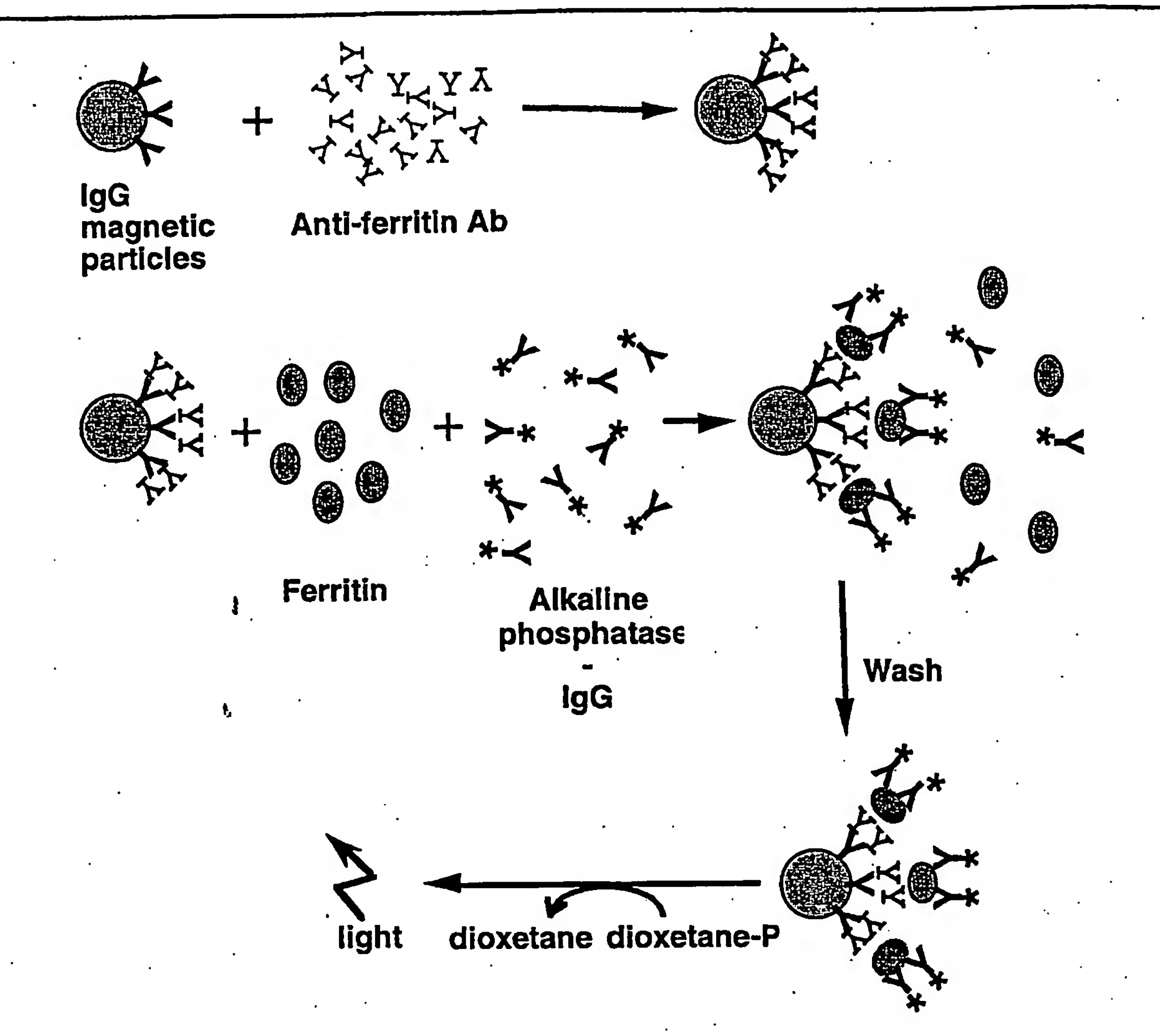


Figure 14 SANOFI Magnetic Microspheres Assay

protoplasts of somatic potato hybrids.[60] They have also been used to collect and concentrate *Chlamydia trachomatis* from urine for subsequent analysis.[61] Cells can be positively or negatively selected using magnetic beads.

Microspheres and Genomes: ECL (enhanced chemiluminescence), robotics, and magnetic microspheres recently have been applied successfully to the human genome project. The microspheres are used in the first step of rapid DNA purification.[62]

Novagen's Straight A's™ mRNA Isolation System uses their Magnetight™ Oligo (dT) Particles– superparamagnetic microspheres covalently coated with oligo $(dT)_{25}$. The protocol is designed to selectively extract and purify mRNA from a variety of sources (Fig. 15). After magnetic separation, the purified mRNA is eluted off the magnetic beads for recovery or for a second round of purification.[63] Promega has a similar isolation procedure.[64]

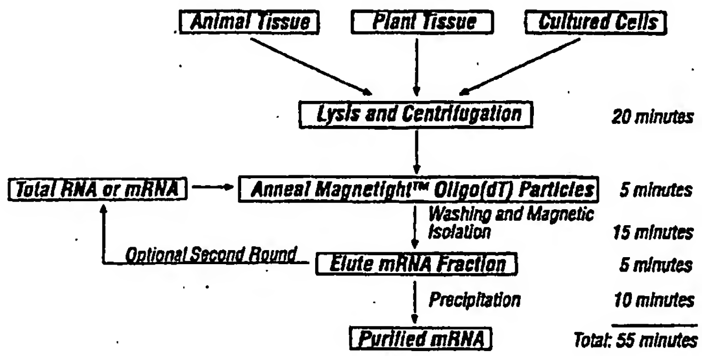


Figure 15 Novagen's mRNA Isolation with Superparamagnetic Particles

Other techniques using magnetic microspheres include oligonucleotide[65] and DNA template purification,[66] "rapid genomic walking,"[67] and sequencing.[68] Wilson used uncoated magnetic particles *twice* to purify ss-DNA– first to collect aggregated M13 phage and later to collect its DNA from ethanol. Magnetic particles are cited as being relatively inexpensive raw materials in a method which reduces labor cost by half.

Streptavidin-coated magnetic particles are also used as a solid support in IGEN's human papilloma virus assay. This is yet another example of a DNA hybrid assay; it is based on PCR (polymerase chain reaction) and read by electrochemiluminescence.[69]

PCR, QBR, LCR– (Q-Beta Replicase and Ligase Chain Reaction) These acronyms relate to molecular amplification techniques used for clinical lab identification of tiny amounts of various infectious agents. All these techniques use solid supports like microspheres and are explained in a good review article.[70] Gene-Trak Systems QBR technique uses "...d(T)-coated magnetic beads, which hybridize with the d(A) tail of the capture probe." The microspheres are used in the QB replication process. In the LCR method, Abbott uses an automated particle capture ELISA with small, protein-coated microspheres, as ImX or AxSym do, to detect the special hapten tags on the ends of the ligated products after sample amplification.

The Human Genome Project is nearing the sequencing stage, and Trevor L. Hawkins of the Whitehead Institute/MIT has developed a method called SPRI (Solid-Phase Reversible Immobilization). DNA is captured onto carboxylate-modified encapsulated superparamagnetic microspheres. After the DNA is bound, the beads are washed with ethanol and then eluted from the beads in a low ionic strength solution. This method enables automatable, high quality DNA template purification, and can be used with all major templates and sequencing enzymes.[66] Richard Guilfoyle (University of Wisconsin) is developing similar protocols.

Microspheres as Markers and Stains: For years, microspheres, especially dyed ones, have been used as tags to identify cells or cell surface antigens on microscope slides. Most useful are those with the color or fluorescent dye *inside* the microspheres.

Because more dye can be loaded inside microspheres than on the surface, the color intensity is greater, and the dyes (especially the fluorophores) are well protected from photobleaching. The microsphere surface properties are not affected, so dyeing does not interfere with protein coating.

photobleaching. The microsphere surface properties are not affected, so dyeing does not interfere with protein coating.

Ab-coated dyed microspheres will stick to cells and identify them (Fig. 16). Dyed ~5µm microspheres, with appropriate Ab coatings, have been used as cell tags in rosette type tests, where the microspheres cluster around certain cells to identify them in the microscope. The new technique called FISH (fluorescent *in situ* hybridization) involves labelling of intact cells using fluorescent microspheres.

Tests

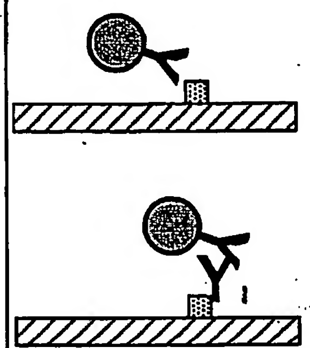


Figure 16 Microspheres as Tags, Markers, or Stains

Zynaxis has an assay method for enumeration of CD4+ and CD8+ T-lymphocytes using mAb-coated fluorescent dyed microspheres and mAb-coated magnetic microspheres. The mixed microspheres form rosettes around the appropriate T-cells. A magnet separates rosettes from unrosetted cells and permits quantitation of fluorescence.[71]

A ciguatoxin test for fish offers a novel use for dyed microspheres as immunomarkers. A roughened wooden paddle is inserted into a cut in the fish. When it is removed, some fish flesh adheres to it. Next, the paddle is dipped in a suspension of antibody-coated, dyed microspheres and washed. A colored paddle shows a positive test for ciguatoxin (Fig. 17). This simple test enables fishermen to keep only safe fish and throw the rest back.[72]

Other applications for dyed microspheres include regional blood flow studies in animals. Multicolored 10 and 15µm microspheres, injected into an animal's circulatory system, become lodged in the tissues during circulation. After tissue biopsies, the colored or fluorescent spheres are recovered and counted or analyzed for size and fluorescence intensity. The E-ZTrac products from Interactive Medical Technologies Ltd. and other similar systems by Triton Corp. and Molecular Probes are examples of these products, and they are replacing radiolabelled microspheres in this field.[73]

Roche Molecular Systems' new "Ultra Direct" technique for processing plasma with exceptionally low HIV-1 titers, involves high-speed centrifugation, followed by lysis of the virions and direct PCR amplification. 0.2 µm red microspheres are added to the sample and spin down with the virions, greatly improving visibility of the pellet.[74]

Plain and dyed microspheres are used as standards for flow cytometry. There is growing interest in "designer" microspheres– dyed with "fluorochromes", "fluorophors" (fluorescent dyes with particular spectral properties), and scintillators. Often only a

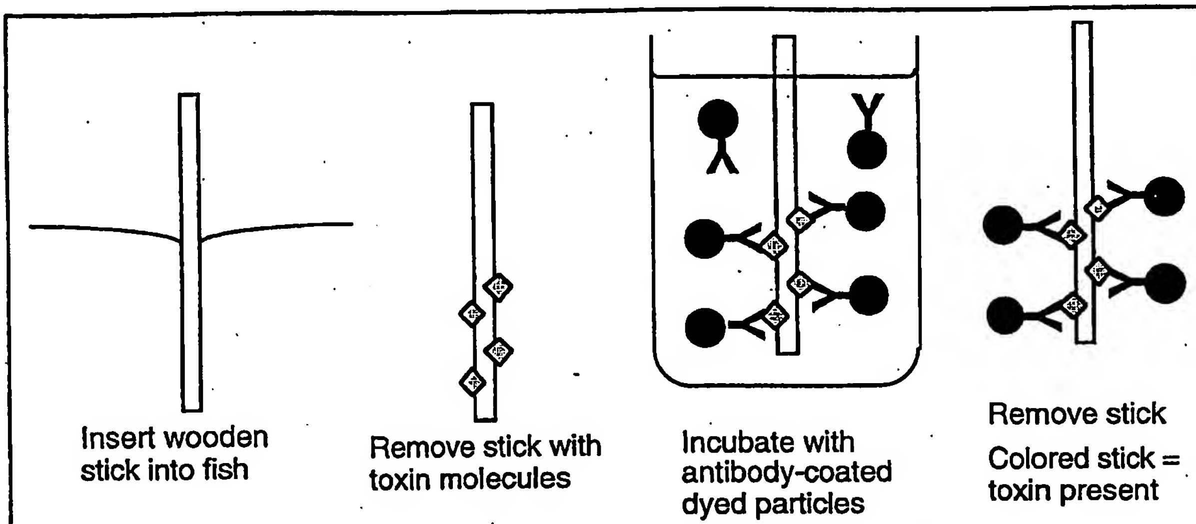


Figure 17 Dyed Microspheres Ciguatoxin Test for Fish (Y. Hokama)

small amount of these dyes is required to give an intense signal. Stains and flow cytometry are obvious applications of these microspheres, and there will be others.

Molecular Probes has microspheres with several dyes in each, yielding spheres which are excited at one wavelength and which emit at a wavelength far removed from the excitation wavelength. In fact there is a cascading of the excitation and emission wavelengths of a series of dyes, so fluorescent light emission from one dye excites the second dye, *etc.*[75] Thus, it is much easier to separate the two wavelengths for detection, sensitivity can be higher, and interferences are minimized.

Proximity Assays: Amersham's scintillation proximity assay (SPA) system uses one microsphere coated with a γ-emitter radio-labelled Ag and another microsphere dyed with scintillator and coated with Ab. When the microspheres are mixed together, an Ag-Ab reaction binds the microspheres together, and light is given off when γ-rays emitted from the Ag-coated microspheres enter the Ab-coated, scintillator-dyed microspheres. When a sample is added to the mixture, any free Ag in the sample will interfere with the two microspheres coming together and decrease light output (Fig. 18).[76]

Similar in concept to SPA, Syva's new homogeneous immunoassay format, Luminescent Oxygen Channeling Immunoassay (LOCI), uses microspheres to measure TSH at 4 attomol! When antibody-coated microsphere pairs are brought together by antigen, molecular oxygen is released by a photosensitizer in one bead and diffuses to the other bead, which contains a high-quantum-yield chemiluminescent receptor. Light emitted corresponds to sandwich formation and is proportional to Ag concentration.[77,78]

Tosoh has a similar idea: Ab_1 and a fluorescer molecule are bound to one particle; Ab_2 and a quencher molecule are bound to another particle. The fluorescer will light unless the quencher is brought close by Ag in the sample, agglutinating or forming a sandwich between Ab_1 & Ab_2. Diminishing light signal is proportional to Ag content.[79]

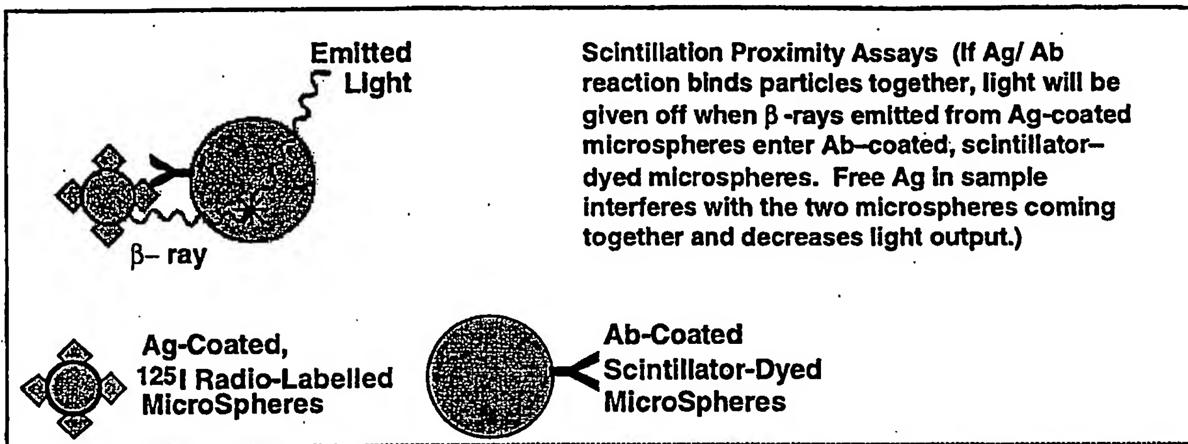


Figure 18 Scintillation Proximity Assay

Summary and Future:
There is a wide selection of existing ways to use particles in diagnostics from LAT's to DNA probe assays. New developments continue to promise an exciting future.

- An environmental consideration has been added to the equation from Germany– the Topfer Decree. Soon manufacturers will be responsible for all plastic packaging. And perhaps all solid waste will be returned to the *manufacturer* for proper disposal. In the diagnostics business, for instance, it appears that plates and tubes will be returned to the supplier. It may be, however, that *liquid* reagents, including dispersed particles (if not otherwise hazardous) will be ordinarily disposable – so "no problem" for particles, in liquid form. This directive is already being seriously considered in planning for new clinical systems.

- Some researchers predict that many immunoassays will soon be done on cells and single microspheres in flow cytometry instruments, as use of these instruments becomes more widespread.[80] Microsphere-based assays run in flow cytometers are being actively studied.[81] Antibody-coated microspheres are mixed with sample. If antigen is present, the laser light is scattered (or the microsphere fluoresces) differently. The difference in light scattered (emitted) by microspheres with and without Ag will be used to quantify the antigen (Fig. 19).

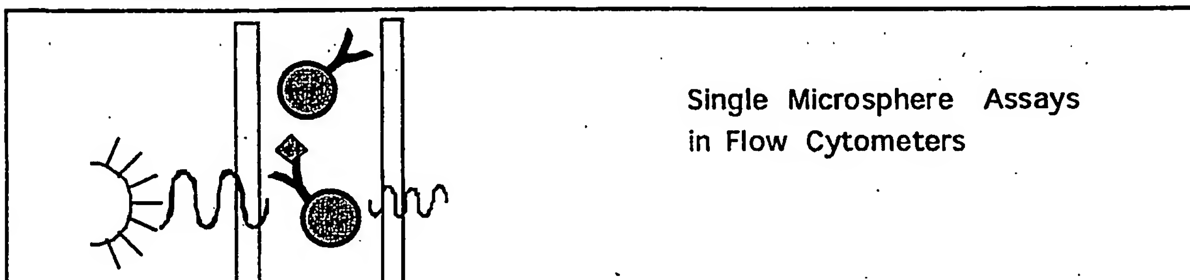


Figure 19 Single Microsphere Immunoassays Done in Flow Cytometers

- *Latex Test for Latex?* An example of these single microsphere assays is one for natural latex *proteins* (NLP), found in impure natural latex products. NLP's can cause

severe allergic reactions in many spina bifida cases, certain other patients, and health workers who are often exposed to, and have become sensitized to, rubber products like shunts and gloves. An NLP Ab assay was constructed using microspheres with NLP coupled to the surface. In use, the microspheres were mixed with suspected samples containing NLP Ab's, then biotinylated IgE was added, followed by avidin-labelled fluorescein isothiocyanate (FITC). Microspheres were put through the flow cytometer and fluorescence measured after activation with 488nm light. Fluorescence is directly correlated with NLP Ab levels.[82]

Some *New* Ideas?

- Try agglutination tests using silica microspheres. More hydrophilic and higher density (~2g/mL) than PS, they will yield different kinds of tests and assays.
- Try coagglutination tests by mixing different sizes of microspheres together or microspheres with bacteria or blood cells. Some novel formats are likely to result.
- Try to make *whole blood* tests using black microspheres. The black microspheres should be visible even in the presence of red blood cells, unless the RBC's interfere somehow with the agglutination.
- "Clear-to-cloudy" test: If very small ($\leq$50 nm) microspheres are diluted to ~1% solids, the suspension is transparent; after agglutination to clumps >300 nm, the clumps are large enough to scatter light, and the suspension becomes turbid. This phenomenon could lead to a simple (OTC?) test: a change of appearance from clear to cloudy signaling a positive result.
- The field of biosensors has been "promising" for several years. An excellent review of immunosensors appeared recently in *Clinical Chemistry*.[83] These promises may soon be delivered and microspheres may be able to help in amplifying the signal from optical and electronic based sensors. For example, Battelle has a "biorefractometer" based upon the Mach-Zehnder approach, which depends upon the difference between two laser light paths, one blank and one with an antibody coating. Analyte binds to the Ab and changes the phase of the light in this path. Microspheres might be used to amplify this difference.
- Similarly, try microspheres as amplifiers of the signal in the new evanescent-wave-based sensor technology (Fig. 20). Ideally, on an antibody-coated biosensor, if antigen in a sample is bound by the antibody, there will be a detectable signal change. However, if the signal is not strong enough, one can add a second antibody, perhaps with a microsphere attached. Then, surely, there will be a large change in the signal, with amplification brought about by the attached microspheres. For example, a press release from Fisons (UK) for their new IAsys biosensor system with evanescent field technology built into the cuvettes claims it can analyze "a wide range of sample types and even cell or particle suspensions."

  Piezoelectric/microsphere immunosensors are now possible. The signal from an Ab-coated piezoelectric crystal will change dramatically when Ag-coated particles are captured on its surface. Free Ag in a sample would probably not create as large a signal change on binding to the same surface, but free Ag would inhibit particle binding and thus moderate the signal change. A sandwich format should also work (Ab/crystal + Ag + Ab/particles gives large signal change; no Ag , no signal change). [Inspired by a recent paper.[84]] Dr. Ben Feldman of UCSF reports, "The idea has

been tried before with limited success."[85,86] AAI-ABTECH also claims to have a pending patent application in this area.

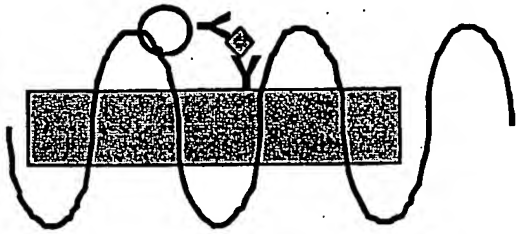


Figure 20 Possible Use of Microspheres in Biosensors

- "Third Wave Inhibition Assay": There may be other ways to detect particle coupling like SPA, LOCI, and the Tosoh concept. Imagine two microspheres dyed with different dyes–"Fluorophore 1" (F_1), excited by a laser at λ_1 and emitting at λ_2 and "Fluorophore 2" (F_2), excited at λ_2 and emitting at λ_3. If one microsphere is coated with Ab, and the other is coated with Ag, and if an Ag/ Ab reaction binds the microspheres together, then F_2 in the Ab-coated (2nd) microsphere will emit light of λ_3 only if excited by λ_2 radiation, emitted by F_1 in the adjacent Ag-coated (1st) microsphere. If there is competing Ag in a sample mixed with the two particles, then the particles will not get together, and no light will be emitted by the second particle.[87] (Fig. 21) But, can these particles get close enough together to turn on the light?

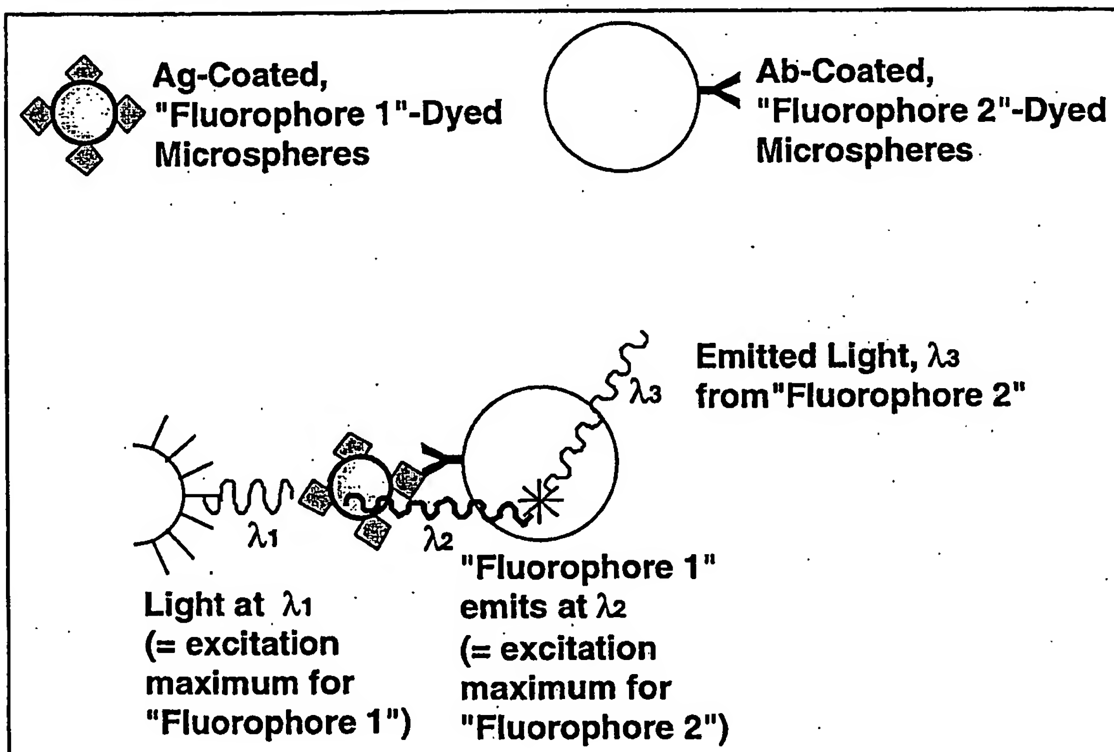


Figure 21 "Third Wave" Assay

- A Wall Street Journal article about Affymax work on DNA detection described chopping a patient's single stranded DNA into ~50 fragments and tagging it with fluorescent dye.[88] Why not use *fluorescent-dyed microspheres* to amplify the signal?

- One researcher has covalently linked a magnetic microsphere (or handle) to one end of a DNA molecule and a 10 µm surface modified polystyrene microsphere (or anchor) to the other end. She then pulls the molecule around with a magnet to stretch it out so she can measure the DNA molecule length and sequence with an atomic force microscope. Similarly, others using optical traps or "laser tweezers" have attached two polystyrene microspheres as handles (one at each end) to stretch out a single actin filament and bring it into contact with a third, silica microsphere coated with myosin molecules to measure the interaction of myosin and actin.[89,90] Others have manipulated silica microspheres in similar displacement experiments.[91]

- "Nanotechnology" (Oak Ridge Conference, 1994) dealt with micromachines, and similar very small applications of clinical chemistry and immunoassays,[92] such as the use of ~6 µm microspheres to demonstrate and test effectiveness of a 5 µm micromachined filter.[93] Similarly, ACHEMA (Frankfurt) 1994 had a session on Microtechnology (analysis in small volumes and instruments) and "nanotools" are being discussed in the trade press.[94] *If the world needs micromachines, then we will have the micro-ball-bearings to keep them running smoothly!*

This is only a beginning!

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